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Review

Localized, non-viral delivery of nucleic acids: Opportunities, challenges and current strategies

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ABSTRACT

Localized delivery of drugs is an emerging field both with regards to drug delivery during disease as well as in tissue engineering. Despite significant achievements made in the last decades, the efficient delivery of proteins and peptides remains challenging, especially in cases requiring long-term release of proteins after application. The localized delivery of nucleic acids (NA) represents an interesting alternative due to higher physicochemical stability of NA, increased efficiency by harnessing cells as bioreactors for the production of required proteins and improved versatility with regards to expression of specific proteins through plasmid DNA or repression of gene products through siRNA. However, unlike most proteins and peptides, NA must be delivered to the cytoplasm or nucleus to be efficacious, resulting in significant delivery challenges. We herein describe frequently used non-viral vectors for the delivery of NA including polyplexes, lipoplexes and lipopolyplexes and summarize recent developments in the field of nucleic acid delivery systems for local application based on hydrogels, solid scaffolds and physical delivery methods. The challenges associated with the different approaches are identified and options to address these challenges are discussed.

1. Introduction

The efficient delivery of nucleic acids (NA) is one of the most exquisite challenges to formulation scientists nowadays. Unlike small chemical drugs and most biologic drugs, NA must be delivered into the cytoplasm or into the nucleus to be effective. Small interfering RNA (siRNA) and antisense oligonucleotides interact with mRNA and therefore must be transported to the cytoplasm. However, transport of NA across the cell membrane is challenging due to its negative charge and high molecular weight [1]. Plasmid DNA (pDNA) additionally requires translocation into the nucleus where it is transcribed into mRNA, eventually resulting in protein synthesis.

From a physicochemical and formulation point of view NA are almost ideal drug molecules. They possess high stability against both chemical and physical degradation, which is

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exemplified by the possibility to extract and sequence DNA from fossils of extinct species and the remarkable half-life of DNA of 521 years [2,3]. It is therefore not surprising that several studies investigating the stability of formulated NA yielded promising results, especially when nucleic acids were protected from hydrolytic and enzymatic degradation, e.g. through freeze-drying [4–6]. It is also important to note that NA potentially require significantly less formulation development work once an optimal formulation has been found compared to other macromolecular drugs such as proteins and peptides. The relatively simple structure of NA results in minimal changes of physicochemical properties of NA when changing the sequence. In contrast, exchange of a single amino acid may result in significantly changed structure and physicochemical properties in the case of proteins and peptides [7,8]. Therefore, it is reasonable to assume that reformulation efforts to fulfill the needs of NA with a new sequence will be minimal. Furthermore, NA, especially siRNA and antisense oligonucleotides can be simply produced by chemical synthesis with good yield and in high purity. In contrast, production of proteins such as monoclonal antibodies or growth factors requires a cellular expression system and extensive purification and/or refolding [9]. Finally, NA possess high specificity, resulting in expression of a specific protein (plasmid DNA) or inhibition of a specific target gene (siRNA, antisense oligonucleotides), potentially maximizing the ratio of desired to undesired effects compared to alternative therapies [10].

Apart from the numerous advantageous properties of NA, the requirement for delivery into the cytoplasm or nucleus represents a significant challenge. This challenge is addressed using attenuated viral vectors as delivery systems or non-viral delivery strategies. A discussion of the general advantages and disadvantages of viral versus non-viral vectors is beyond the scope of this review and the reader is referred to the review by Guo et al. for detailed information on this topic [11]. We herein focus on non-viral delivery strategies, which are often preferred because of safety concerns associate with viral vectors and versatility and ease of modification of non-viral vectors [11].

In most studies, systemic administration of NA delivery systems through the parenteral route is investigated, primarily because of its versatility, e.g. with regards to treatment of distant or disseminated cells, mostly in the context of tumor therapy. However, achieving efficient delivery of NA to target cells after parenteral application requires overcoming numerous extracellular and intracellular barriers. Stability and compatibility of the carrier system in the blood stream must be maintained to achieve prolonged circulation, allowing the delivery system to reach its target site. This entails surface modification with hydrophilic polymers to avoid uptake of the delivery system by the reticulo-endothelial system (RES), crosslinking to prevent premature release of the NA cargo and control of delivery system size to avoid blocking of capillaries and to allow extravasation at the target site, e.g. in the tumor or liver [12,13]. Additionally, active or passive targeting strategies may facilitate the efficient extravasation and cellular uptake at the site of action. In recent years, stimuli-responsive systems have been developed, further improving accumulation of the delivery system at the site of action. These systems change their physicochemical properties in response to extracellular stimuli such as changes of the pH value, enzymatic environment etc. [14,15]. It is therefore obvious that the successful development of NA delivery systems for systemic administration requires an optimal combination of different properties within the delivery system, representing a significant formulation challenge. Furthermore, the clinical application of these delivery systems has been significantly limited because of concerns with regards to toxicity of the vehicle and unsatisfactory delivery efficiency.

While the majority of studies has focused on improving the efficiency, stability and compatibility of delivery systems for systemic application, localized delivery of NA represents an interesting but largely untapped alternative, worthy of increased exploration. While it is certainly not possible to achieve the versatility of systemic application, e.g. regarding transfection of distant or disseminated cells, localized delivery avoids several major barriers associated with systemic delivery and is therefore more likely to result in safe and efficacious therapy. Achieving sufficiently high drug concentrations at the target site is a common challenge in drug therapy. In the case of NA delivery this general challenge is aggravated by the necessity of intracellular delivery of NA [9]. Because of the sensitivity of NA towards enzymatic degradation, encapsulation is often required but on the other hand efficient release must be maintained [1]. These requirements increase the complexity of delivery systems, especially in the case of systemic delivery.

Within this review, we seek to give an overview of the current status of localized delivery of NA using non-viral delivery systems. The discussion is however focused to topical delivery in the broadest sense, excluding oral, nasal and pulmonary delivery as these delivery routes have additional special requirements to the delivery system. We shortly portray non-viral vectors that are employed for localized delivery of NA and review recent systems for localized NA delivery based on various matrices. Our aim is to highlight challenges but also opportunities associated with this approach to NA delivery and discuss potential fields of application.

2. Non-viral nucleic acid delivery systems employed for localized delivery

The high molecular weight and negative charge of NA represents a major hurdle to efficient cellular uptake. For most cell types, the size requirement for particle uptake is in the rage of up to 200 nm, significantly smaller than the hydrodynamic diameter of DNA of a few thousand base pairs [16]. Furthermore, negatively charged moieties anchored to the cell membrane result in electrostatic repulsion of negatively charged NA. Non-viral delivery agents are designed to improve cellular uptake efficiency either through complexation and charge reversal of NA or through physical methods allowing NA to directly enter the cell. However, the challenge with such delivery systems is on the one hand to prevent the NA degradation within the micro- or nanoparticles and on the other hand to achieve efficient intracellular release [1].

2.1. Polymeric systems (Polyplex)

Cationic polymers such as polyethylenimine (PEI), chitosan or dendrimers are among the most studied non-viral vectors for NA [17–20]. These polymers proved to be highly efficient in vitro, and several are commercially available as transfection agents (JetPEI, Polyplus transfection SA; ExGen500, Biomol GmbH).

Positively charged polymeric nanocomplexes (polyplexes) in the size range of tens to hundreds of nanometers are spontaneously formed when NA and suitable cationic polymers are mixed under appropriate conditions. Polyplexes facilitate cell attachment through electrostatic interaction, internalization by endocytosis and endosomal escape [18,21,22]. One of the main advantages of polymeric systems for non-viral delivery is their ease of modification, allowing straightforward design and synthesis of multifunctional delivery systems [11]. Some of these modifications include the shielding of surface charge through modification with hydrophilic polymers prior to [23] or after complex formation [24] with NA. Furthermore, conjugation of targeting moieties can be performed in a similar fashion either directly [25] or via grafted hydrophilic polymers [26]. Biodegradable crosslinking, e.g. using bioreducible linkers, is another potential modification allowing tailoring the stability of polyplexes in different environments to specific needs [27,28]. However, cytotoxicity of polycations is one of the major problems, which was frequently found to correlate with transfection efficiency [29,30]. Several groups addressed this issue, e.g. through bioreducible crosslinks in the polymer backbone, resulting in improved degradation and potentially improved excretion [31,32]. A detailed overview of the current status of polymeric non-viral vectors can be found in [33] and advantages and disadvantages of polymeric non-viral vectors are discussed in [17].

2.2. Lipid-based systems (Lipoplex)

Lipoplexes are formed through the interaction of anionic NA with the surface of cationic liposomes, resulting in aggregated and multilamellar complexes. The challenges associated with cationic liposomes as a vector for NA delivery are their high zeta potential, low transfection efficiencies and often an inefficient protection against lysosomal NA degradation [4]. Lipid-based systems were among the first non-viral vectors used to transfer NA into cells. Felgner et al. showed in 1987 that N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), a synthetic cationic lipid, is able to condense plasmid DNA (pDNA) and to transfect different cell lines in vitro [34]. The complexation efficiency with pDNA was found to be close to 100% and it was observed that complexes fuse with the cell membrane, resulting in efficient intracellular delivery of the cargo [34,35]. The interaction with the cell surface is thought to be primarily due to electrostatic interaction of positively charged lipoplexes with negatively charged residues at the cell surface, such as sialic acid. In addition, hydrophobic interactions contribute to fusion with the cell membrane and cargo delivery. In this regard, the addition of neutral helper lipids like dioleoylphosphatidylethanolamine (DOPE) or cholesterol results in stabilization of liposomes and improved membrane fusion and hence improves transfection efficiency [36]. Later studies found that efficient transfection depends on clathrin-mediated endocytosis and direct translocation of NA across the cell membrane is of only minor importance [22,37]. However, similar to the polymeric systems, cytotoxicity is a major concern. Toxicity was frequently found to be associated with the charge ratio between NA and cationic lipid, the dose administered and the type of reagent or cell type [38]. One way to deal with these toxicity issues is the local administration of lipoplexes, reducing the effects on non-target cells. Numerous improvements and modifications to the basic concept of lipofection as described above have been published, including stabilized liposomes, immunoliposomes and stimuli-responsive liposomes. The reader is referred to [39,40] for a detailed discussion of current lipoplex systems and to references [38,39] for an in depth discussion of advantages and disadvantages of lipoplexes as vectors for NA.

2.3. Lipopolyplex

Lipopolyplexes, representing ternary complexes composed of cationic polymers, cationic liposomes and NA, were developed in an attempt to combine the advantageous properties of polyplexes and lipoplexes. Besides improvements with regards to stability in the presence of serum, particle size and reproducibility of transfection, lipopolyplexes were shown to significantly increase transfection efficiency compared to polyplexes [41,42]. Through further supplementation with hyaluronic acid or heparin, lipopolyplexes with very low immunostimulatory potential compared to traditional lipopolyplexes were obtained [43]. Recently, structurally stabilized lipopolyplexes consisting of lipoplexes which were alternatively coated with poly(acrylic acid) and polyethylenimine were prepared [44]. These lipopolyplexes showed improved transfection efficiency and reduced cytotoxicity compared to various polyplexes and lipoplexes and commercial lipofectamine. Lipopolyplexes are therefore an efficient alternative to traditional lipo- and polyplexes with reduced cytotoxicity. Additional information on the advantages and disadvantages of lipopolyplexes can be found in [44].

3. Delivery systems for localized release of nucleic acids

3.1. Local delivery of naked nucleic acids

The direct administration of NA as a liquid is certainly the simplest form of local delivery, it is highly attractive due to simple formulation development and administration and, under appropriate conditions, results in efficient NA delivery. As an example, the treatment of choroidal neovascularization by direct injection of stabilized siRNAs was investigated by Shen et al. [45]. In this study, Sirna-027, a chemically stabilized siRNA against vascular endothelial growth factor receptor 1 (VEGFR1), was administered locally through intravitreal or periocular injections in a murine model of choroidal or retinal neovascularization. Upon injection of Sirna-027, VEGFR1

mRNA levels were significant reduced by 40–57%. Labeled siRNA was detected in retinal cells for 4–5 days after intravitreal injection, siRNA was also able to penetrate the sclera and choroidea after periocular injection and was detectable in the retina for at least 5 days. Finally, treatment with Sirna-027 resulted in efficient suppression of ocular neovascularization in murine models of choroidal and retinal neovascularization. The safety and tolerability of direct intravitreal injection of Sirna-027 was later tested in a phase 1 clinical study in 26 patients with choroidal neovascularization resulting from neovascular age-related macular degeneration [46]. Intravitreal injections of 100–1600 μg of Sirna-027 were well tolerated and no dose-limiting toxicity was observed. Secondary objectives including retinal thickness and visual acuity were found to be stabilized or improved after treatment, respectively. Further phase 1 and phase 2 studies evaluating stabilized siRNA targeting the RTP801 gene were performed in patients with neovascular age-related macular degeneration [47] and diabetic macular edema [48], respectively. Similar to the study on Sirna-027, intravitreal injections of siRNA in these two studies were generally well tolerated and safe. Only few mild to moderate treatment-related adverse events were observed. However, Kleinman et al. demonstrated that inhibition of VEGF expression and reduction of neovascularization might be due to unspecific activation of cell surface Toll-like receptor 3 (TLR3) [49]. In addition, it was shown that cellular uptake of siRNA was not required for this effect. These observations as well as failure to meet efficacy requirements in clinical studies resulted in discontinuation of the development of ocular injection of Sirna-027.

Meuli et al. reported that direct injection of naked plasmid DNA into full-thickness wounds in a murine model resulted in efficient local transfection of macrophages, fibroblasts and adipocytes [50]. Interestingly, transfection efficiency of naked DNA was comparable to efficiency of optimized cationic liposome/DNA complexes. Similar results were obtained in pig skin, in rats and another murine model before [51–53]. Local transfection efficiency after administration of naked DNA in the skin can be further improved through oscillating solid microneedles [54,55].

3.2. *Composite systems*

The development of drug delivery systems for the localized release of NA, apart from application of naked NA described above and physical methods described below, frequently requires the combination of a component that condenses NA and allows the transfection of target cells (vector) and a matrix that controls release of the vector and provides physical support. In the simplest case, NA or NA/vector combinations are locally administered as a solution without the need for a supporting matrix. If a matrix is used, NA may be incorporated into the matrix (encapsulation) or may be attached to the matrix surface (immobilization).

3.2.1. *Hydrogels*

Hydrogels are among the most frequently used systems in the field of localized therapy, including tissue engineering. The well hydrated, tissue-like environment of hydrogels, their variability with regards to the chemical composition and modification as well as the typically mild, aqueous fabrication conditions all render hydrogels ideally suited as scaffolds for localized NA delivery. Hydrogels are typically formed either by crosslinking or by self-assembly from hydrophilic polymers of natural, synthetic or semi-synthetic origin. The release of encapsulated NA can be controlled via the pore size of the hydrogel, its degradation rate or through physical interaction or chemical conjugation of the vector system with hydrogel components. In the following sections, we will highlight the advantages and challenges associated with various hydrogel-based delivery of non-viral vectors. For an in depth discussion of properties and application of natural and semi-synthetic polymers for drug delivery the reader is referred to [56].

3.2.1.1. *Hydrogels based on natural polymers*. Natural polymers like collagen, fibrin or alginate were frequently used to prepare NA releasing hydrogels. Natural hydrogels in this regard may serve as a reservoir for NA vectors with release being controlled by the hydrogel pore size, vector–matrix interactions and/or degradation. Such systems have been developed based on collagen [57–59], fibrin [60,61], alginate [62,63], chitosan [64] and hyaluronic acid [61]. The sustained release of NA from these biocompatible scaffolds frequently resulted in improved transfection efficiency both in vitro and *in vivo* [65,66]. Furthermore, sustained release of polyplexes or lipoplexes was often found to significantly reduce the cytotoxicity of non-viral vectors [59,67]. Retention of the vector within the hydrogel and hence release rate can be modified by variations of the vector and hydrogel chemistry. As an example, Cohen-Sacks et al. modified a collagen hydrogel matrix with poly-L-lysine to improve the retention of plasmid DNA through non-specific electrostatic interactions [57]. Alternatively, NA or vector, i.e. polyplexes can be chemically crosslinked with the hydrogel matrix. Capito et al. showed by direct comparison of a) simple soaking of a pre-crosslinked hydrogel and b) crosslinking of plasmid DNA with the scaffold, that the latter method allows to reduce burst release and to link DNA release to hydrogel degradation [58]. Similarly, control over sustained release of siRNA/PEI complexes was achieved by covalent attachment of PEI via ester linkages to photocrosslinked dextran hydrogel [68]. However, aggregation of non-viral vectors during incorporation into hydrogels associated with loss of activity was identified as a major limitation. Lei et al. recently developed a procedure termed caged nanoparticle encapsulation allowing loading of concentrated non-viral vector nanoparticles into various hydrogels avoiding aggregation [61,69]. The encapsulation process consisted of lyophilization of polyplexes using agarose and sucrose and subsequent reconstitution of the lyophilized powder with hydrogel precursor, followed by crosslinking for hydrogel formation (Fig. 1).

3.2.1.2. *Hydrogels based on synthetic polymers*. While natural hydrogels often excel with regards to biocompatibility, biodegradability and can be obtained from renewable resources [56], synthetic polymers allow simple chemical modification and functionalization, improved control over composition and properties and often carry lower risk of immunogenicity than natural polymers [70]. It is therefore not surprising that numerous hydrogel systems for the delivery of

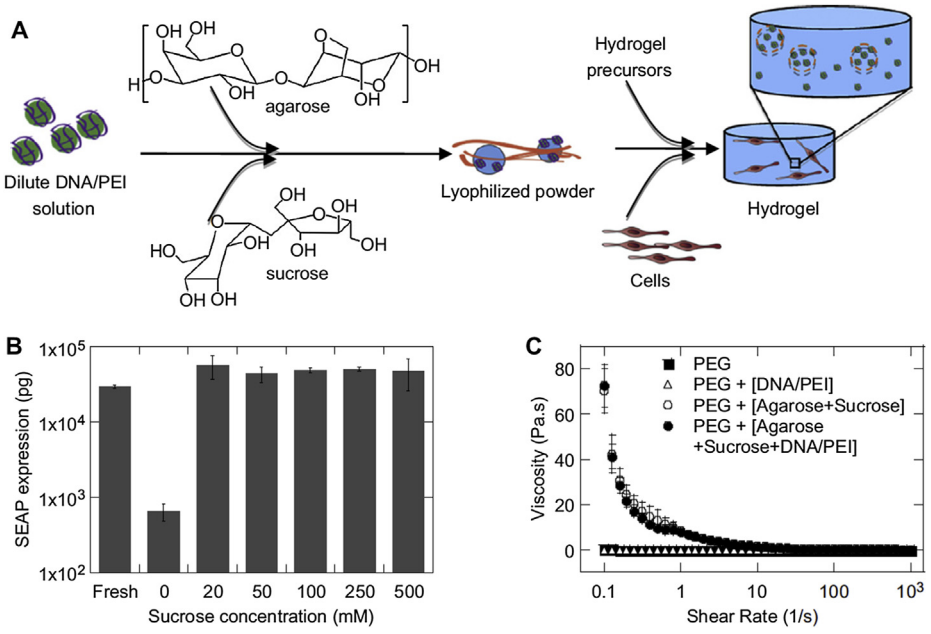

Fig. 1 – (A) Schematic representation of caged nanoparticle encapsulation process: polyplexes consisting of plasmid DNA and PEI were mixed in the presence of agarose and sucrose and lyophilized. The lyophilizate was reconstituted with hydrogel precursor solution and subsequently crosslinked to prepare hydrogels. (B) Transfection efficiency of polyplexes after lyophilization depending on sucrose concentration. Polyplexes containing plasmid coding for mammalian secreted alkaline phosphatase (SEAP) were used to transfect HEK293 cells after lyophilization and transfection efficiency was compared to freshly prepared polyplexes (Fresh). (C) The viscosity of the hydrogel precursor solution was increased through addition of agarosein an attempt to prevent aggregation of polyplexes during gelation. Reprinted from [69] with permission from Elsevier.

NA based on synthetic polymers have been described. As with natural polymers, NA incorporation may be achieved by simple soaking of the hydrogel with NA, encapsulation of vectors during hydrogel formation or physical or chemical conjugation of NA or vector to the hydrogel matrix [71–74]. Furthermore, in situ forming hydrogels [75,76] and stimuli-responsive systems [77,78] have been developed based on synthetic polymers. Release properties may therefore be varied according to the specific needs through the mode of NA incorporation or by variation of scaffold properties. Takahashi et al. varied the crosslinking density of PEG hydrogels to modify the diffusion-controlled release of siRNA/PEI polyplexes [71]. NA release can be sustained even further through conjugation of NA/vector complexes to the scaffold. Li et al. used a triblock copolymers consisting of methoxy-PEG, poly(ε-caprolactone) and poly[2-(dimethylamino)ethyl methacrylate (MPEG-PCL-PDMAEMA) that efficiently condensed plasmid DNA to generate hydrogels by crosslinking mPEG blocks using α-cyclodextrin [74]. Using this supramolecular hydrogel, sustained release of DNA for up to 6 days was achieved.

Efficient cell infiltration is another critical property for hydrogel delivery system employed for tissue engineering and therefore control over the macroporous structure is crucial. Orsi et al. developed hydrogels with variable and controllable pore size distribution based on PEG hydrogels using gelatin microparticles for templating, achieving improved and controllable cell migration into the scaffold [79]. Similarly, matrix stiffness of hydrogels affects NA delivery performance and was shown to be inversely related to transfection efficiency in hydrogels [80,81]. Keeney et al. synthesized hydrogels with a tunable matrix stiffness between 2 and 175 kPa using PEG-dimethacrylate (PEG-DMA) and gelatin-methacrylate (Gelatin-MA) [82]. Cells were coencapsulated with polyplexes inside hydrogels and cell proliferation and transfection efficiency was investigated. While cell proliferation decreased with matrix stiffness, transfection efficiency of optimized polyplexes was found to be high across a broad stiffness range, but being strongly reduced at high gel stiffness (Fig. 2). Therefore it is important to note that besides optimization of vector efficiency and release rate, mechanical and physical properties of the matrix such as porosity and stiffness may be important optimization parameters, especially in case where cell infiltration into the scaffold is intended.

3.2.1.3. Bioresponsive hydrogels. Despite the progress made using hydrogel-based delivery, release of NA vectors for such composite systems is controlled solely by hydrogel and vector properties, such as diffusion- and degradation rate or vector/hydrogel interactions. In contrast, bioresponsive drug delivery systems have been developed in recent years. These systems change their properties in response to a biological trigger such as changes of the pH, temperature, light or presence of biomolecules such as enzymes [83]. Such functions are highly desirable when designing hydrogels for NA delivery, allowing novel modes of drug release, e.g. self-regulating systems that

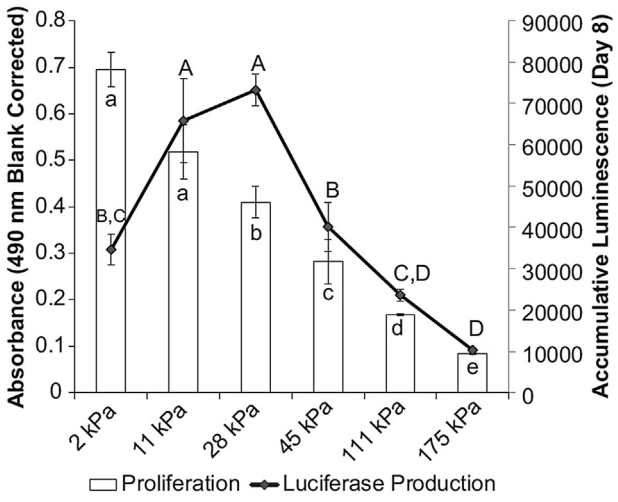

Fig. 2 – Analysis of cell proliferation and transfection efficiency using luciferase as reporter gene of HEK293 cells 8 days after coencapsulation with polyplexes into PEG-DMA/Gelatin-MA hydrogels. Bars or points with shared letters are not statistically different from each other. Uppercase letters apply to luciferase expression. Reprinted from [82], with permission from Elsevier.

release the drug only in a well-defined disease state. Among the numerous bioresponsive systems described to date, hydrogels that release their payload in the presence of elevated levels of enzymes will be discussed in more detail. Cell-matrix interactions represent an interesting trigger for drug release from hydrogels, especially for tissue engineering applications. Matrix metalloproteinases (MMPs) are enzymes that degrade both, matrix and non-matrix proteins, and are important for remodeling of the extracellular environment of cells [84]. MMP-degradable hydrogels support cell growth and allow cell migration and have been intensively studied by Hubbell and coworkers and others [85–87]. The significant potential of MMP-degradable hydrogels as drug delivery matrix was recently highlighted by Purcell et al., incorporating recombinant tissue inhibitor of MMPs (rTIMP-3) into MMP-sensitive hydrogels [88]. MMP activity in a porcine model of myocardial infarction was significantly reduced after targeted delivery of hydrogel/rTIMP-3 and left ventricular remodeling was attenuated. However, both the loading capacity of hydrogels as well as the stability of proteins within hydrogels is limited, representing a significant hurdle to efficient, long-term treatment in many fields. Therefore the targeted, stimuli-responsive delivery of plasmid DNA or siRNA through MMP-sensitive hydrogels may represent an attractive alternative, allowing long-term bioavailability since cells infiltrating the scaffold act as bioreactors for the required proteins. NA delivery from MMP-degradable hydrogel matrices has been recently investigated by Tatiana Segura and coworkers [78,80,89]. In these studies, it was shown that hydrogels loaded with non-viral vectors and allowing cell infiltration by MMP-sensitive crosslinking resulted in long-lasting transgene expression in combination with low cytotoxicity.

3.2.2. *Porous structures*

In contrast to hydrogels, microporous structures as discussed herein are solid, porous or foam-like matrices typically composed of polymers like polyurethans or poly(lactic-co-glycolic acid) (PLGA) and intended for implantation or topical application as opposed to injection. Such scaffolds may be prepared through a variety of methods, including top-down approaches such as simple foam forming and electrospinning, or bottom-up approaches such as microparticle composites. In the following paragraphs we will focus on foam-like structures and electrospun scaffolds, representing frequently applied technologies. One of the main challenges associated with solid porous structures for localized NA delivery is to achieve both, optimal cell infiltration as well as appropriate release of NA. To fulfill the requirement of optimal cell infiltration, structures of sufficiently high porosity are fabricated. On the other hand, such highly porous structures are frequently characterized by very thin walls separating the pores, which results in short diffusion distance and fast release if drugs are simply encapsulated into the scaffold. However, despite these challenges, solid structures allow for substantial variation of material composition, structure and surface modification and because of this flexibility are highly interesting for the development of multifunctional NA delivery systems.

3.2.2.1. *Foam-like scaffolds*. Biodegradable polyurethans (PUR) are promising foam-like biomaterials with proven performance as injectable drug delivery system for various small chemical and biologic drugs [90–94]. PURs suitability for NA delivery has recently been evaluated by Nelson et al. [95,96]. SiRNA was complexed into nanoparticles using a diblock copolymer composed of 2-(diethylamino)ethyl methacrylate, butyl methacrylate and 2-propyl acrylic acid and nanocomplexes were encapsulated into the matrix during PUR foam preparation. These scaffolds were shown to release nanocomplexes over 3 weeks based on a diffusion-controlled mechanism and bioactivity of siRNA was maintained for 4–21 days depending on the model used [95,96]. The therapeutic potential of PUR scaffolds was evaluated *in vivo* after subcutaneous implantation of scaffolds into balb/c mice. SiRNA targeting prolyl hydroxylase domain protein 2 (PHD2) was encapsulated into scaffolds as described above. During normoxia PHD2 activity results in degradation of pro-angiogenic hypoxia inducible factor 1α (HIF1α). Suppression of PHD2 therefore results in enhanced activity of HIF1α, mediating expression of pro-angiogenic vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), and others [96]. After 14 days, VEGF and FGF-2 expression increased approximately 200% and 300%, respectively and a significantly improved development of functional vascular structures was imaged and quantified by micro-CT (Fig. 3), providing preliminary evidence for therapeutic efficiency of such scaffolds *in vivo*.

An alternative approach was followed by Rives et al., applying a sandwich approach where the structure of the polymeric material was varied in an attempt to uncouple scaffold structural requirements from requirements of drug delivery [97]. The authors prepared layered poly(lactic-co-

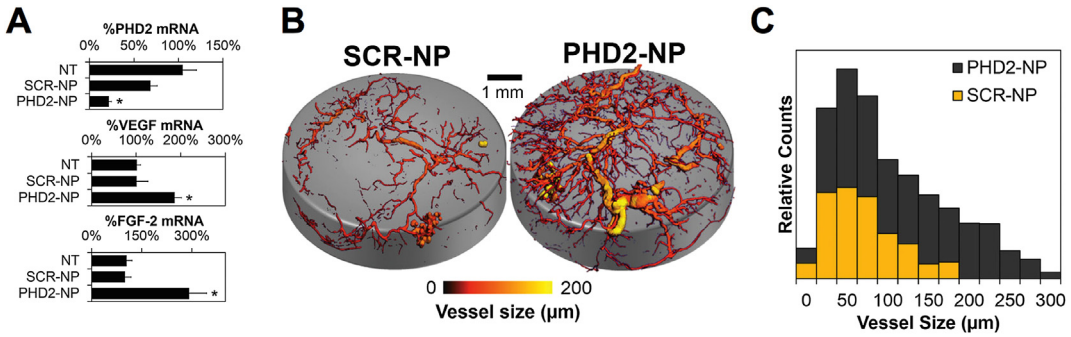

Fig. 3 – Delivery of siRNA directed against PHD2 increases expression of VEGF and FGF-2 and improves neovascularization. A) PHD2 was efficiently silenced through application of PHD2-siRNA loaded nanoparticles (PHD2-NP) whereas scrambled siRNA (SCR-NP) had no significant effect versus non-treated (NT) cells. Silencing of PHD2 resulted in significantly increased expression of VEGF and FGF-2. B) Micro-CT imaging revealed improved vessel formation after application of PHD2-NP vasculature. C) Analysis of micro-CT images showed a significant increase of both vessel number and vessel size for PHD2-NP scaffolds compared to application of scaffolds loaded with scrambled siRNA. Reprinted from [96] with permission from Wiley.

glycolic acid) (PLGA) scaffolds consisting of porous layers allowing cell infiltration, and non-porous layers containing plasmid DNA and allowing slow release. The non-porous layer was fabricated from PLGA microparticles and loaded with plasmid DNA. It was then sandwiched between two porous layers, which were fabricated from PLGA microparticles using gas foaming in combination with particle leaching. These scaffolds allowed efficient cell infiltration into the outer porous layers. Pronounced burst release of plasmid DNA was observed within the first 3 days, resulting in declining transgene expression after 3 days. However, after implantation of scaffolds loaded with plasmid DNA encoding FGF-2 into intraperitoneal fat of C57BL/6 mice, a significant increase in the total vascular volume fraction was achieved, in part being a result of an increase in vessel size. Nevertheless, achieving more sustained plasmid release kinetics would potentially extend transgene expression and reduce plasmid-associated inflammatory response [97].

Finally, additive manufacturing (also known as 3D printing or rapid prototyping) is another approach followed to fabricate scaffolds with just the right combination of properties. In contrast to traditional scaffold manufacturing methods rapid prototyping allows the fabrication of complex structures composed of different materials and does not require the use of toxic solvents. Andersen et al. recently presented scaffolds composed of an NA-loaded hydrogel component and polycaprolactone for structural stability fabricated by additive manufacturing [98]. The hydrogel composition was optimized to achieve printability by hydroxyethylcellulose, stability by alginate and cell attachment and survival by hyaluronan and the gels were loaded with siRNA and used for 3D printing as shown in Fig. 4.

In order to improve load-bearing ability of scaffolds, hydrogels were co-printed with PCL fibers, resulting in scaffolds that were impossible to crush by hand. These composite scaffolds could be seeded with stem cells and encapsulated siRNA was shown to stay localized and being taken up by nearby cells. Image analysis further revealed that localized knockdown of green fluorescent protein (GFP) used as reporter gene could be achieved in checker patterned scaffolds with alternating patches with siRNA targeting GFP and mismatched siRNA (Fig. 5) [98].

3.2.2.2. *Electrospun matrices*. The structural similarity of electrospun nonwovens to the extracellular matrix and their wide variability with regards to composition and dimension of individual fibers as well as structure of nonwovens have resulted in significant attention on the electrospinning technique in the biomaterials and tissue engineering fields [99]. Numerous polymers, such as PLGA, PCL and PEG as well as various fabrication techniques such as emulsion electrospinning, coaxial electrospinning or two-nozzle electrospinning may be used and combined [99]. Furthermore, subsequent modification of the fiber surface opens additional options for the fabrication of complex composite materials. Herein we will highlight some recent developments in the field of electrospun nonwovens for nucleic acid delivery, focusing on novel techniques that go beyond diffusion-based release of NA from electrospun polymer fibers representing the first generation of electrospun NA delivery systems [100–102]. Burst release of large amounts of loaded NA was a major challenge associated with some of these early systems [100,101]. However, this problem could at least partly be solved through encapsulation of NA/polycation complexes into nonwoven scaffolds [102,103]. Saraf et al. prepared core/shell fibers using PCL, PEI and hyaluronic acid (HA) as shell material, while PEG and DNA were located in the core of the fibers [103]. The effects of variation of four processing parameters, on fiber diameter, release kinetics of PEI-HA and transfection efficiency were evaluated based on a fractional factorial design. The average transfection efficiency was affected by PEG molecular weight and concentration and lasted for up to 60 days under optimized conditions in vitro. An alternative approach to encapsulation of NA into polymer fibers was presented by Sakai et al., who used a layer-by-layer assembly to load NA onto the fiber surface [104]. In this study, plasmid DNA and PEI were employed as polyanion and polycation, respectively to achieve successive deposition and

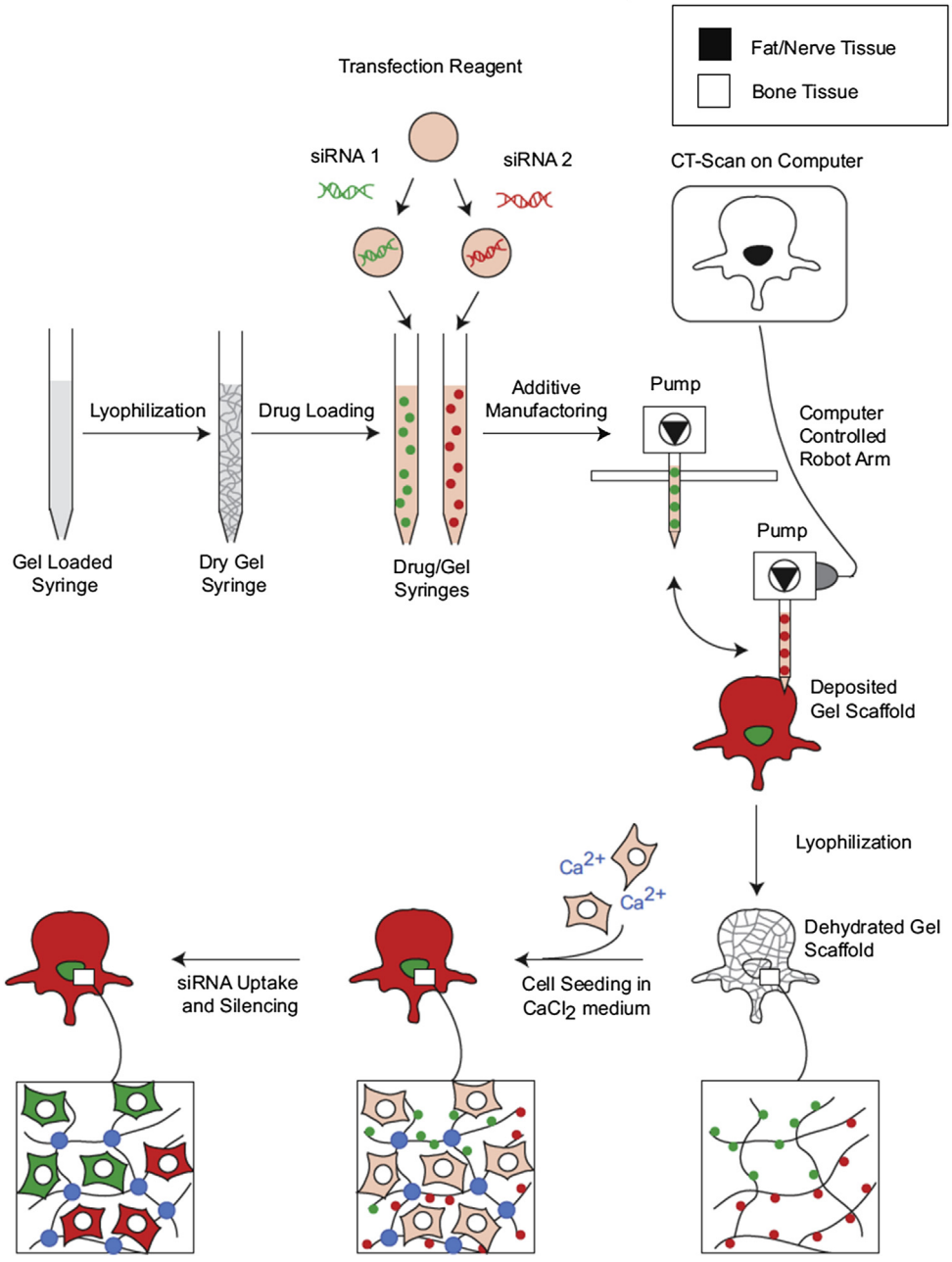

Fig. 4 – Schematic representation of scaffold manufacturing procedure using additive manufacturing approach. Reprinted from [98], with permission from Wiley.

multilayer buildup. Transfection efficiency of scaffolds prepared using this coating technique depended both on the amount of DNA loaded onto the scaffolds as well as on incubation time with cells.

Interestingly, several studies reported that significant cytotoxicity of polyplexes was observed despite encapsulation or adsorption of polyplexes to electrospun nonwovens, which was at least in part attributed to significant burst release [100–102]. In a recent study the cytotoxicity and transfection efficiency of polyplexes encapsulated into the innermost or the outermost layer of microcapsules consisting of silk fibroin was directly compared in vitro [105]. From these experiments it became evident that encapsulation of polyplexes significantly reduced cytotoxicity while maintaining transfection efficiency. Furthermore, both, cytotoxicity and transfection efficiency were reduced when polyplexes were incorporated into the innermost layer compared to polyplexes being present in the outermost layer. One might therefore conclude that despite a significant reduction of cytotoxicity of polyplexes can be achieved through encapsulation and sustained release, delivery systems must still be optimized to achieve a proper balance of transfection efficiency versus cytotoxicity.

Recent reports showed that electrospun scaffolds are quite suitable for designing bioresponsive NA delivery systems

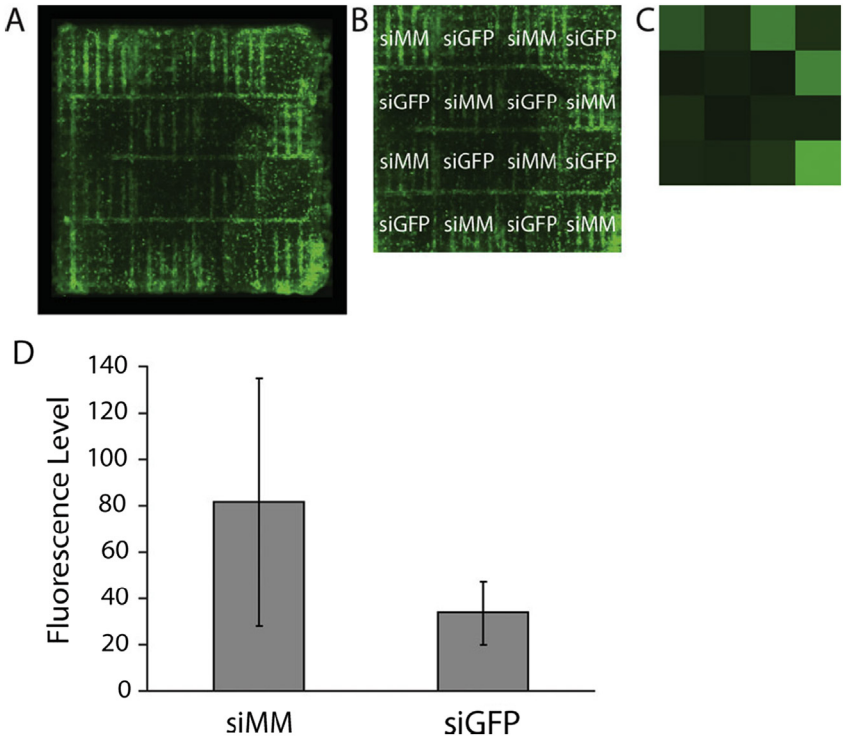

Fig. 5 – Checker patterned scaffolds consisting of PCL fibers and hydrogel containing siRNA directed against GFP or mismatched siRNA. A) GFP expressing human mesenchymal stem cells were seeded into the scaffold and imaged at day 3 using a laser fluorescence scanner. B) The resulting image was cropped and centered on the scaffold. C) Bilinear downsampling to 4 × 4 pixels representing 4 × 4 siRNA patches was performed. D) The fluorescence level of each pixel represented by its color value was determined and is shown as mean and standard deviation of 8 samples for each group. Reprinted from [98], with permission from Wiley.

[106–109]. Kim et al. developed a strategy where PEI was coupled via an MMP-sensitive linker onto the surface of electrospun fibers consisting of PCL-PEG copolymer [106,108,109]. Upon incubation with plasmid DNA or siRNA, efficient complexation of NA with surface-bound PEI was observed and MMP activity resulted in release of PEI/DNA complexes and bioresponsive transfection (Fig. 6). The therapeutic efficiency of this approach was evaluated in an *in vivo* model of diabetic ulcers, either using plasmid DNA encoding for human epidermal growth factor (hEGF) [108] or employing siRNA targeted at MMP-2 [109]. In both cases, accelerated wound recovery was observed, either through increased expression of hEGF leading to enhanced re-epithelialization or through reduction of MMP-2 expression resulting in normalization of the wound milieu. These studies show the potential of bioresponsive delivery systems based on electrospun scaffolds with regards to achieving and maintaining just the right drug concentration in response to and controlled by a specific disease state. Further developments will likely focus on improved protection of NA during storage and after application, increased loading capacity for NA and simplified synthesis and fabrication of these systems.

Finally, electrospun scaffolds allow the combination of various delivery systems and drugs within one device as well as significant structural variability. The ability to combine different NA within one scaffold is exemplified by studies conducted by Li and colleagues who coencapsulated plasmid DNA coding for VEGF (pVEGF) and FGF (pFGF) into core/shell fibers by emulsion electrospinning [110,111]. In a first study the core contained plasmid DNA/PEI polyplexes while the shell consisted of poly(DL-lactide)-poly(ethylene glycol) (PELA) [111]. The release kinetics of plasmid DNA from fibers containing only pVEGF was very similar to fibers containing both plasmids and both types showed low burst release of approximately 10% (Fig. 7A). Cytotoxicity of polyplexes released from scaffolds was evident but was reduced when polyplexes were encapsulated into the core of the fibers compared to fiber mats where polyplexes were adsorbed to the surface (Fig. 7B). The authors also observed that cell proliferation after approximately 3 days was more pronounced for scaffolds with encapsulated polyplexes than for adsorbed polyplexes. This was attributed to sustained release of plasmid, resulting in long-lasting transfection and increased growth promotion through the expressed growth factors.

Despite the fact that these scaffolds successfully promoted the generation of blood vessels *in vivo*, low cell viability, pronounced inflammation and necrosis lead to the development of a system using calcium phosphate/DNA (CP/DNA) nanoparticles instead of PEI/DNA polyplexes [110]. It was assumed that the transfection efficiency of plain CP/DNA nanoparticles decreases over time due to aggregation and growth of nanoparticles, finally resulting in particles too large for cellular

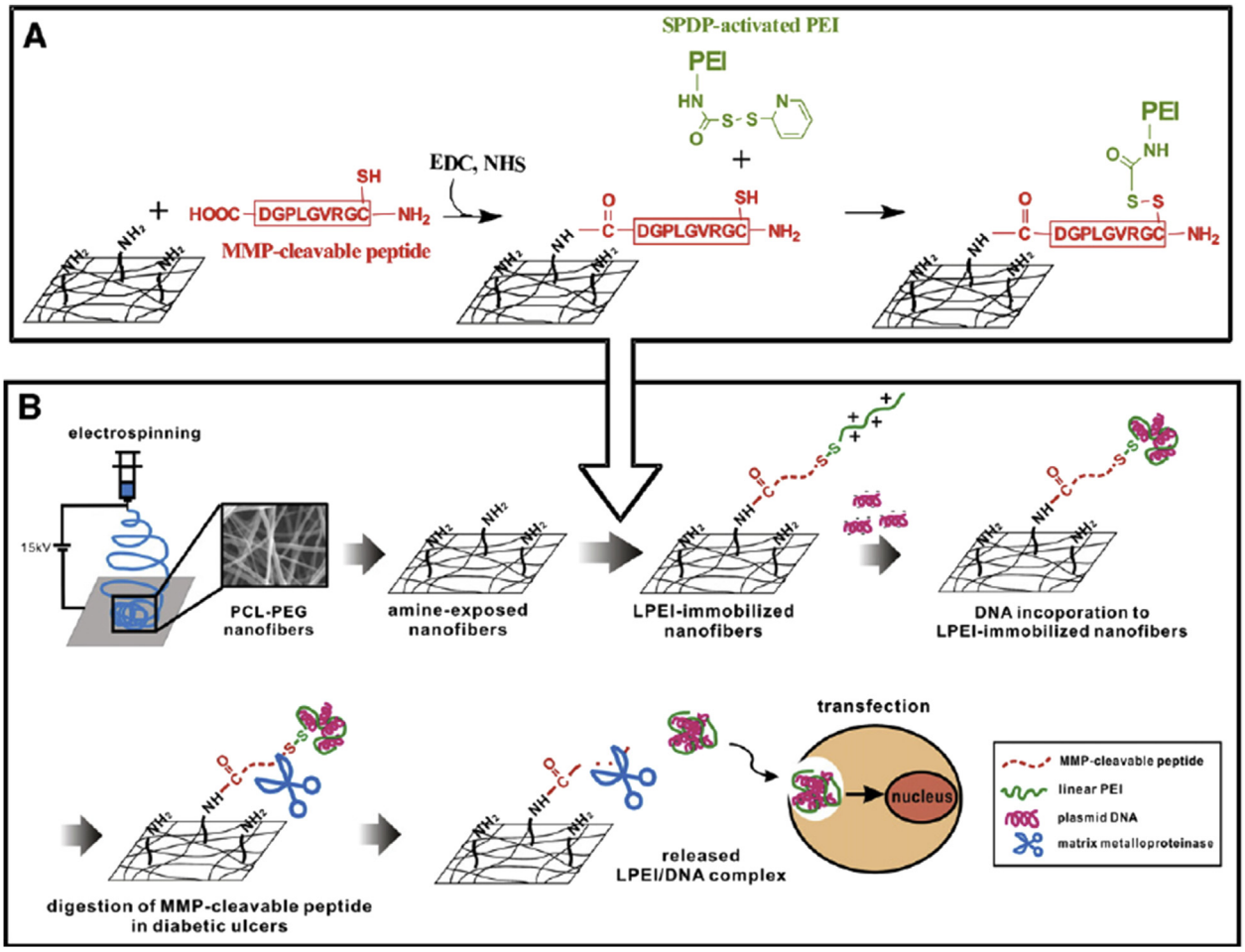

Fig. 6 – Schematic presentation of MMP-sensitive immobilization of PEI on the surface of electrospun fibers. A) Synthesis of PEI-grafted PCL-PEG fibers: an MMP-sensitive peptide linker was conjugated with free primary amines on the fiber surface through 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry, followed by conjugation of N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)-activated PEI to free terminal cysteine of the peptide linker. B) Schematic presentation of fabrication of the drug delivery system and bioresponsive release. Reprinted from [106], with permission from Elsevier.

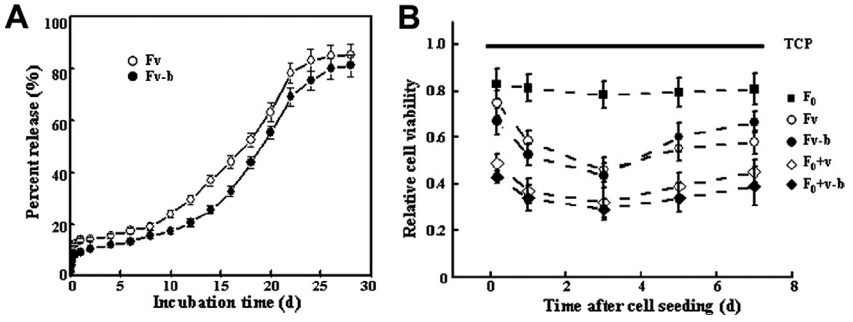

Fig. 7 – A) In vitro release of plasmid DNA from electrospun fibers containing pVEGF alone (Fv) or combined pVEGF and pFGF (Fv-b). B) Cell attachment 4 h after seeding and cell viability at different time points after incubation of HUVEC with plasmid DNA polyplexes encapsulated into fibrous mats Fv and Fv-b, or plasmid DNA polyplexes infiltrated into empty fibrous mats with pVEGF alone (F0+v) or both pVEGF and pFGF (F0+v-b) compared to empty fibrous mats (F0) and tissue culture plastic (TCP). Reprinted from [111], with permission from Elsevier.

uptake [112]. Li et al. hypothesized that encapsulation of CP/DNA nanoparticles into electrospun fibers might alleviate this phenomenon. CP/DNA nanoparticles indeed significantly reduced cytotoxicity and inflammation reaction compared to PEI/DNA polyplexes. Furthermore, the combined encapsulation of pVEGF and pFGF was shown to be more effective with regards to the density of newly formed blood vessels *in vivo* than delivery of individual plasmids [110].

3.3. *Physical methods*

Besides polymer- and liposome-based non-viral systems, several physical methods have been applied for the localized administration of NA in an attempt to improve safety, efficacy and targeting. Physical approaches to NA delivery include hydrodynamic methods, iontophoresis, microneedles, ultrasound and electroporation. The latter two methods are most frequently applied and are described in more detail in the following sections.

3.3.1. *Ultrasound*

Ultrasound facilitated delivery (sonoporation) of nucleic acids is probably the physical method, which is most often applied for localized delivery. Sonoporation relies on the transient formation of pores in the cell membrane in the presence of microbubbles, resulting in efficient uptake of various molecules into the cells [113]. Microbubbles have been used as ultrasound contrast agents for several decades and various formulations are commercially available and FDA approved for human use (e.g. SonoVue® from Bracco International, Optison® from Mallinckrodt and Definity® from Lantheus Medical Imaging). However, besides use as a contrast agent, these microbubbles have also been recognized as drug delivery vehicles for non-permeable molecules such as macromolecular drugs and NA [114,115]. Deshpande and Prausnitz showed in vitro that the combination of sonoporation using Optison microbubbles and PEI/DNA complexes significantly improved transfection efficiency over each of the treatments alone [116]. Similarly, improved cytoplasmatic uptake of lipoplexes through cell membrane pores was observed [117]. Ultrasound facilitated delivery was also successfully used for siRNA transfection with naked siRNA [118], siRNA-liposome complexes [119] and siRNA-polycation complexes [120]. These studies point towards potential improvements of NA delivery efficiency *in vivo* using ultrasound. Already in 2003, Pislaru et al. found that ultrasound exposure enhanced gene delivery efficiency in rat skeletal muscle after intramuscular injection of microbubbles mixed with naked plasmid DNA coding for luciferase compared to the same formulation without ultrasound application [121]. A dose-related increase of ultrasound facilitated transfection efficiency was observed and transfections through sonoporation were spatially highly restricted compared to control experiments with adenoviral gene transfer (Fig. 8). Interestingly, significant differences in transfection efficiency in different muscles were observed, which were unexpected and attributed to flaws inherent to IM injection and varying doses per volume in the different muscles [121].

Another approach to utilizing sonoporation for the spatially restricted transfection of tissues was presented by Kowalczuk et al. [122]. In this study, transfection of the ocular ciliary muscle with naked DNA in combination with microbubbles and ultrasound was investigated. Similar to the study by Pislaru et al., significantly improved gene expression was observed after ultrasound treatment. Despite a rise of lens and ciliary muscle temperature during the treatment, no damage to the tissue was observed. Unfortunately, the study was performed using reporter genes only (luciferase, green fluorescent protein (GFP) and β-galactosidase) but nevertheless shows that expression of therapeutic proteins in the ciliary muscle is potentially feasible.

Ziadloo, Xie and Frenkel recently presented an alternative approach to gene delivery through sonoporation [123]. In contrast to many studies in the field, no microbubbles were used to improve ultrasound efficiency. Instead, pulsed, focused ultrasound (pFUS) was applied immediately prior to intratumoral injection of naked tumor necrosis factor alpha (TNF-α) plasmid DNA. Exposure to pFUS alone had no effect on tumor growth compared to the control group. Injection of TNF-α plasmid DNA alone (without pFUS treatment) resulted in significant tumor growth inhibition, which was further significantly improved after pFUS treatment in combination with TNF-α plasmid. Histological evaluation of the tumors revealed larger necrotic regions and improved distribution and penetration of fluorescent surrogate nanoparticles after pFUS treatment. The authors therefore conclude that besides the structural effects of ultrasound treatment that resulted in an increase of the pore size of the tissue and enhanced distribution of the plasmid, the enlarged space between cells could have also improved the hydraulic conductivity. This would result in increased fluid flow from the tumor core to its periphery and further contribute to improved penetration and distribution of the injected solution (Fig. 9).

3.3.2. *Electroporation*

The permeability of the cell membrane for non-permeable, macromolecular drugs can also be increased through application of an external electrical field. Electroporation has several advantages compared to polymeric and liposomal transfection vectors: a) high transfection efficiency in primary cells is achievable, b) fewer safety concerns are associated with this method, c) electroporation is easy to perform, and d) efficiency is only marginally influenced by the cell line used [124]. Over 30 years of research on electroporation [125] have led to technologically quite advanced methods with several systems being commercially available. Electroporation has been tested in numerous clinical trials, providing evidence that the method can be successfully applied in clinical therapy [126]. Due to the wealth of reports available and decades of research, we herein focus on selected recent studies and otherwise refer to the reviews by Gotthelf and Gehl [127] and Wells [128] for detailed information on electroporation.

The basic mechanism of siRNA electroporation was recently investigated on single-cell level by Paganin-Gioanni et al. [129]. In this study, electrotransfer of NA was directly observed by confocal microscopy, providing evidence for the direct transfer of siRNA across the cell membrane. Electropermeabilization occurred on the two opposite sides of the cell facing the electrodes, whereas siRNA entry was found to

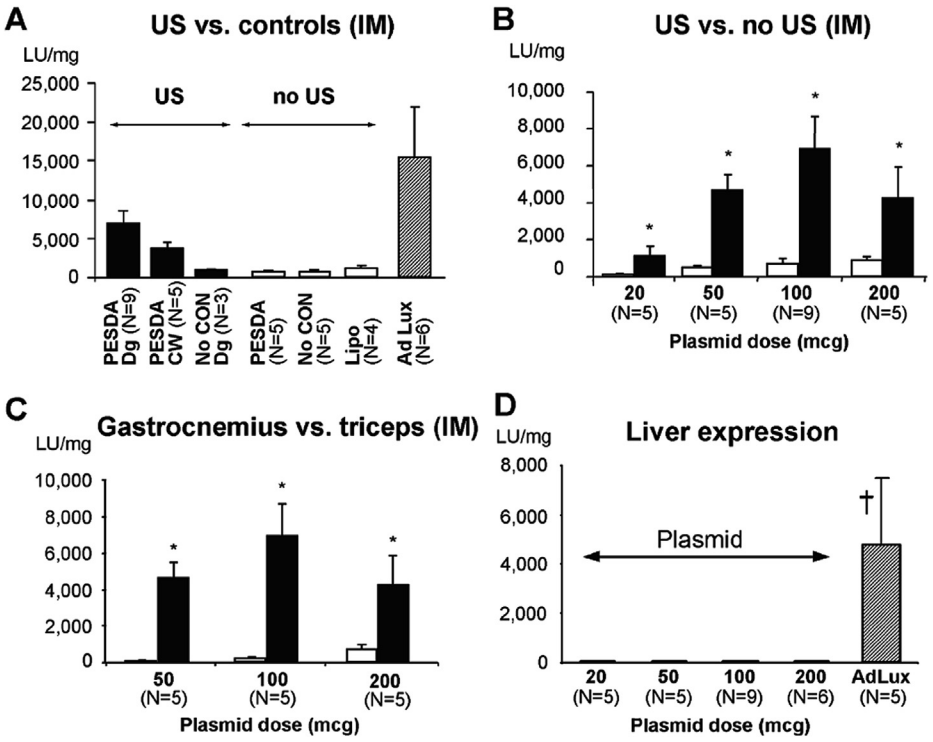

Fig. 8 – A and B: Reporter gene expression 3 days after i.m. injection of luciferase plasmid into the triceps brachii. A: Effect of ultrasound (US) treatment on transfection efficiency of different vectors. From left to right: plasmid + perfluorocarbon exposed sonicated dextrose albumin (PESDA) + diagnostic US; plasmid + PESDA + continuous wave US; plasmid + diagnostic US; plasmid + PESDA, no US; plasmid alone, no US; plasmid + LipofectAmine®, no US; adenovirus, no US. B: Dose-response of plasmid sonoporation in the presence of PESDA (filled bars) vs. plasmid alone without US (open bars). C: Transfection efficiency in gastrocnemius (open bars) and in the triceps brachii (filled bars) at different dose levels. D: Luciferase expression in the liver after treatment with different doses of plasmid + PESDA + US (filled bars) and adenovirus (striped bar). *$P < 0.05$ vs. corresponding open bar; †$P < 0.05$ vs all filled bars. Reproduced with permission of Oxford University press from [121].

occur only on the side facing the cathode, suggesting that electrophoresis aids in electrotransfer. Interestingly, the mechanism of siRNA electrotransfer was found to be somewhat different from plasmid DNA transfer. While siRNA appeared to rapidly and freely penetrate the cell membrane during electroporation, plasmid DNA first formed long-lived spots on the plasma membrane and translocation into the cytoplasm occurred in a second step several minutes after electroporation took place.

Electroporation is well established for the transfer of DNA and extensive studies provided both optimized electroporation devices as well as – parameters. However, fewer studies have investigated methods for optimal RNA delivery. Knowing that the basic mechanisms of electroporation-enhanced NA transfer on a cellular level differ between DNA and RNA, it might be hypothesized that optimal transdermal transfer conditions might be widely disparate. Broderick et al. studied *in vivo* transdermal electroporation for both DNA and siRNA [130]. It was shown in this study that a) low voltage electroporation (10 V range) elicited the most robust transfection for plasmid DNA and b) the same parameters applied for plasmid DNA resulted in successful electrotransfer of siRNA. Overall, no site inflammation or tissue damage was observed using this procedure confirming the good tolerability of low voltage electroporation.

From a patient perspective, topical electroporation-enhanced NA delivery might appear inconvenient due to the necessity of intradermal injection followed by more or less invasive electroporation. Lee et al. therefore developed a monolithic hybrid system consisting of a dissolving microneedle and an electrode to combine both steps within the same device (Fig. 10) [131]. In contrast to NA-coated metal microneedles, this system allows the transfer of significantly larger amounts of NA into the skin, leading to improved therapeutic efficiency. The dissolving microneedles were produced through antidromic isolation by drawing lithography from maltose using the aluminum electrode as drawing pillar and were loaded with plasmid DNA coding for luciferase. Insertion of this device into the skin bypasses the stratum corneum and releases DNA into the interstitial space of the skin. Electroporation can be performed directly through the electrode. Using these hybrid microneedles, subcutaneous tumors were treated with plasmid DNA containing the proinflammatory cytokine interleukin 12 in a mouse model. Hybrid electro-microneedles were most efficient at suppressing tumor growth and with regards to survival after tumor inoculation.

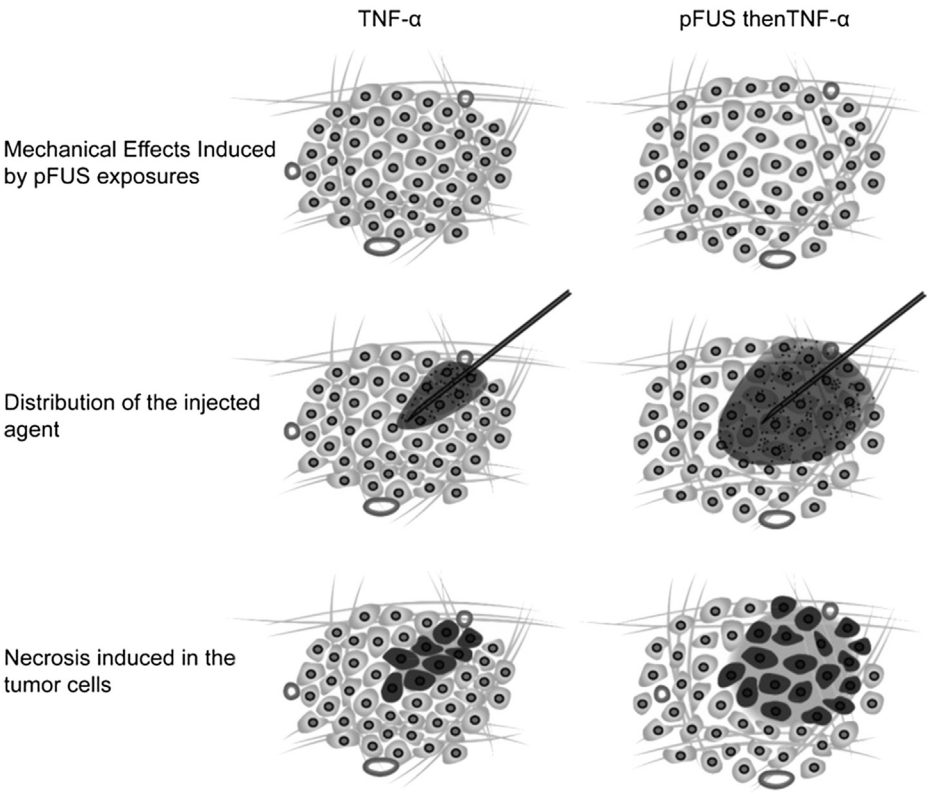

Fig. 9 – Schematic representation of the effect of pFUS on transfection with TNF-α plasmids. Top: Mechanical changes such as enlarged space between parenchymal cells are induced by pFUS treatment. Middle: These mechanical effects improve penetration and distribution of injected plasmid solution. Bottom: Therefore, a larger region of necrosis is observed, ultimately leading to reduced tumor growth. Reprinted with permission from [123], Acoustic Society of America.

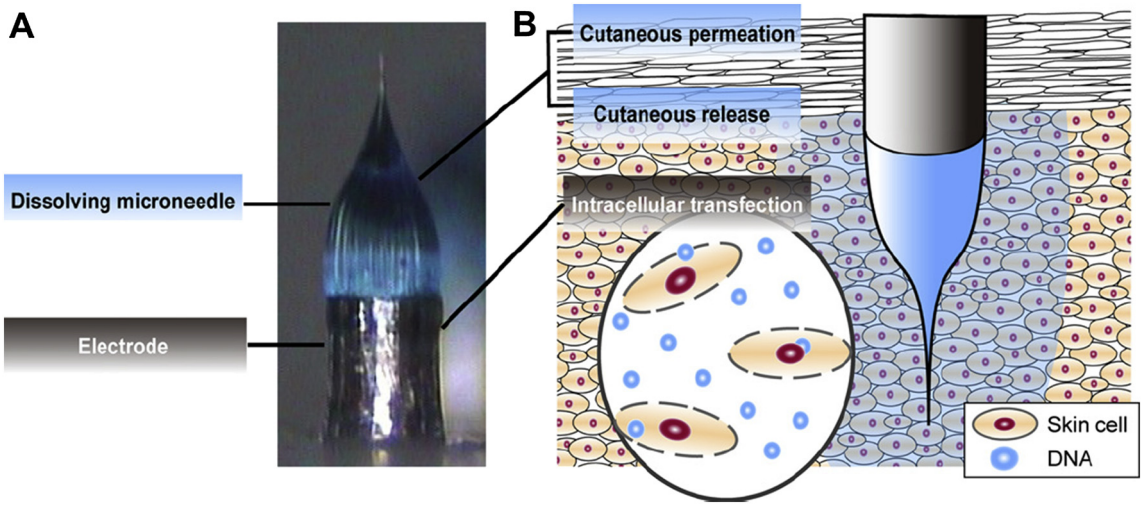

Fig. 10 – Schematic representation of (A) hybrid electro-microneedles consisting of an electrode pillar made from aluminum and a dissolvable microneedle made from maltose. (B) Hybrid electro-microneedles are inserted into the skin, allowing permeation of the stratum corneum and releasing DNA into the interstitium. Electroporation is directly performed using the aluminum electrode. Reprinted from [131] with permission from Elsevier.

4. Conclusions and outlook

Numerous approaches to local delivery of NA have been published in recent years. Besides drug delivery systems intended for topical or localized therapy, tissue engineering has been a main driver for research and development. Without reiterating the basic considerations as described in the introduction, it should be emphasized here that for applications where the drug delivery system is in direct contact

with the body for an extended duration, NA delivery might be a promising alternative to protein or peptide delivery due to higher stability of NA and the ability to employ resident cells as bioreactors. Significant progress has been made in the past decades in the development of non-viral vectors for gene delivery, which forms the basis for scaffold-based NA delivery. However, systemic administration of non-viral vectors is still associated with problems such as insufficient blood half-life, inefficient targeting and cytotoxicity, all of which can be avoided or alleviated by localized delivery.

The discontinuation of the clinical development of naked siRNA for treatment of choroidal and retinal neovascularization was a setback for localized NA delivery in the eye and the discovery of non-specific, TLR3-mediated effects of siRNA has questioned the therapeutic suitability of naked siRNA. Nevertheless, both the indication as well as the delivery route are still highly interesting. To improve siRNA specific effects, further research into drug delivery systems allowing protecting siRNA from degradation and unspecific recognition and with the ability to facilitate efficient cellular uptake is necessary.

The development of bioresponsive, enzyme sensitive delivery systems might result in a major improvement of drug therapy as these systems add a new dimension to controlled drug release. Current systems mainly focus on improved cell migration within hydrogels facilitated by MMP-sensitive crosslinks. However, some studies showed that MMP-sensitive peptides may also be used to control drug release, e.g. resulting in release of MMP inhibitors specifically at elevated MMP levels. Advanced electrospun drug delivery system might in the future use this concept for the self-regulated control and normalization of the wound milieu, while at the same time providing mechanical support and protection from contamination by bacteria.

Finally, physical methods for NA delivery such as electroporation have been continuously developed for decades and are highly versatile with regards to the type of NA used. The combination of advanced electroporation techniques with specialized microneedle arrays represents a clinically feasible, relatively simple and potentially safe approach to dermal delivery of NA. While the range of applications of such systems is limited compared to hydrogels or electrospun nonwovens, their simplicity, the improved control over device structure and the well-defined location of NA delivery might represent significant advantages with regards to clinical evaluation and regulatory acceptance.

Localized NA delivery therefore is a promising strategy for the safe and efficacious treatment of numerous diseases. Further development of non-viral vectors, nano- and microstructured scaffolds and NA derivatives is expected to result in significant improvements with regards to controlled NA release, biocompatibility of scaffolds and transfection efficiency.

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